

INHIBITION OF BLOOD COAGULATION FACTOR XII: EVALUATION OF FLAVONOIDS AS A DRUG CANDIDATE

by
Betsy Alice Crosswhite

A thesis submitted to the faculty of The University of Mississippi in partial
fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
March 2018

Approved by

Advisor: Dr. Ziaeddin Shariat-Madar

Reader: Dr. Hoang V. Le

Reader: Dr. Greg Tschumper

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ACKNOWLEDGEMENTS

Foremost, I would like to thank my advisor and mentor Dr. Ziaeddin Shariat-Madar for his continual efforts to expand the boundaries of my academic experience. I would also like to thank Dr. Hoang V. Le and Dr. Greg Tschumper for their time and contribution as readers. Finally, I offer thanks to the Sally McDonnell Barksdale Honors College for providing a community in which I have been able to experience intellectual and personal growth.

ABSTRACT

Betsy Crosswhite, Dr. Ziaeddin Shariat-Madar
University of Mississippi Pharmacy School, Dept. Biomolecular Sciences, University,
MS 38677

This study investigated natural product inhibitors of blood coagulation Factor XII (FXII). FXII initiates the Kallikrein Kinin-System (KKS) within the intrinsic coagulation pathway. Increased expression of this system leads to proinflammatory and procoagulant activities. Inflammation occurs through downstream activation in the KKS. Following the activation of FXII-dependent pathway, bradykinin (BK) binds to the BK receptor 2 (B₂) and produces inflammatory signaling. Abnormally increased BK can result in life threatening Hereditary Angioedema (HAE), characterized by severe reoccurring swelling. FXII is critical for pathogenic thrombosis, but is dispensable for hemostasis. This unique property of FXII makes it an attractive drug target. We hypothesized that the identification of a specific inhibitor for activated FXII could lead to the reduction of Hereditary Angioedema Type III (HAE III) symptoms and hinder thrombosis formation without an increased risk for bleeding. In this study, we presented an extension of our previous work, which dealt with high-throughput screening of plant extracts and a large compound collections to target FXIIa. We identified myricitin as a candidate compound via a systemic experimental screening. Chromogenic activated FXII activity assays were preformed to test the effects of myricitrin analogues on plasma enzymes (FXIIa, FXIa, and kallikrein). To assess the inhibitory effects of candidate compounds on these proteases, the release of paranitrianalide from the chromogenic substrates were measured

as indicator of FXIIa, FXIa, or kallikrein activity. The results of this study showed that myricetin, an analogue of myricitrin, significantly inhibited FXIIa at the sub-micromolar to the low micromolar concentration range. Overall, myricetin has the potential to be a useful multifunctional therapeutic drug in patients with HAE and thrombolytic diseases.

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LIST OF ABBREVIATIONS

FXII.....	Factor XII
FXIIa.....	Alpha Factor XII
PK.....	Prekallikrein
KAL.....	Kallikrein
HK.....	High Molecular Weight Kininogen
BK.....	Bradykinin
B2R.....	B2 Receptor
HAE.....	Hereditary Angioedema
C1NH.....	C1-esterase inhibitor
VTE.....	Venous Thromboembolism
aPTT.....	A prolonged activated partial thromboplastin time
TF.....	Tissue Factor
Myr.....	Myricitrin
pNA.....	Para-Nitroaniline

INTRODUCTION

1.1 Factor XII-Driven Plasma Contact System

Fibrin formation functions through two pathways: extrinsically through tissue damage or intrinsically initiated by Factor XII (FXII). The Kallikrein Kinin-System functions within the intrinsic coagulation pathway supporting proinflammatory and procoagulant activities¹.

The multifunctional FXII initiates the coagulation pathway². FXII is released by the liver and mapped on the human chromosome number 5³. It is divided into two regions, one heavy chain and one light chain. The heavy chain is divided into three distinct regions. The first region has a molecular weight of 40,000, can bind to negatively charged surfaces and does not detect enzymatic activity⁴. The second has a molecular weight of 28,000, cannot participate in artificial surface binding⁴. Similar to the first heavy chain region, the third can securely bind to negatively charged surfaces, with a molecular weight of 12,000⁴. FXII activation occurs either by auto activation by contact with a negative surface (solid-phase activation), or by adding a protease that leads to enzymatic cleavage (fluid-phase activation)⁵.

Zymogen FXII goes through a conformational change to its activated enzymatic form, FXIIa, following contact with a negatively charged surface⁶. Local activators include platelet polyphosphates, mast cell-derived heparin, collagen¹, and systemic initiators such as: glycated proteins, microparticles, and activated endothelium¹.

FXIIa cleaves prekallikrein (PK) which subsequently forms kallikrein⁷. Activated PK

can reciprocally activate FXII and induce a positive feedback loop for FXII activation⁷ (Diagram 1). Kallikrein cleaves high molecular weight kininogen (HK) which results in the liberation of bradykinin (BK). BK then binds to the kinin B2 receptor (B2R) which activates proinflammatory signaling pathways⁸. The Kallikrein Kinin-System affects coagulation, vascular permeability through inflammation and edema, blood pressure, glucose homeostasis, adipogenesis, and wound healing⁹ (Diagram 2).

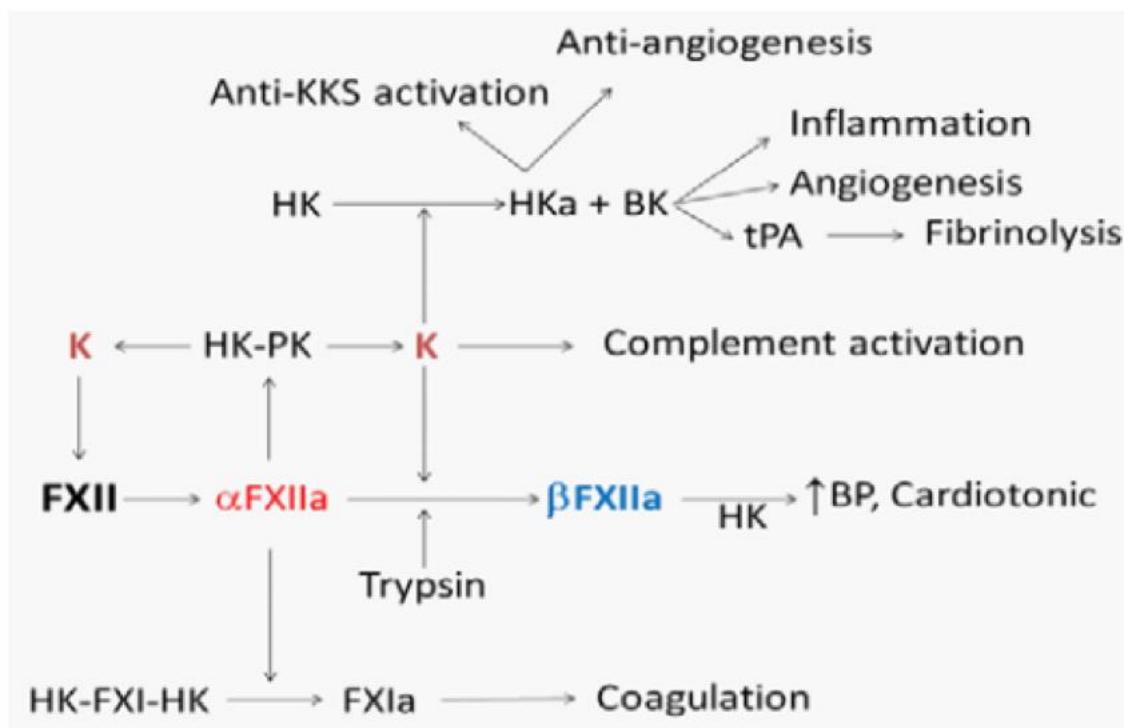


Diagram 1: The Mechanism of Activation behind coagulation Factor XII.

Lynch J, Shariat-Madar Z (2012) Physiological Effects of the Plasma Kallikrein-Kinin System: Roles of the Blood Coagulation Factor XII (Hageman Factor). J Clin Toxicol 2:e105.

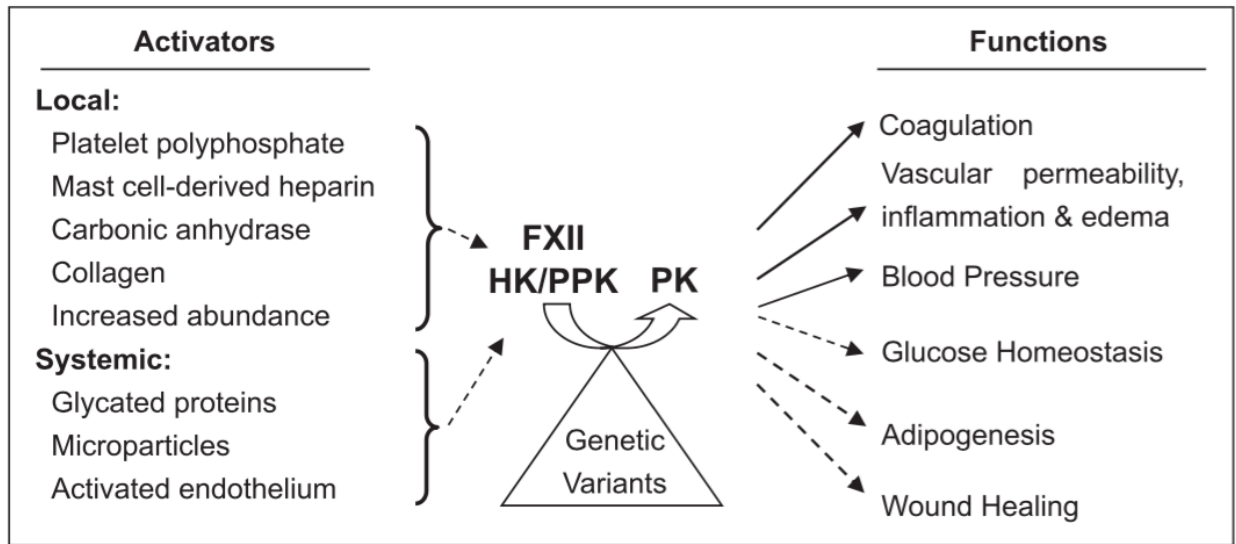


Diagram 2: The Kallikrein-Kinin system can be activated both locally and systemically by FXII. Feener, E. P.; Zhou, Q.; Fickweiler, W. Role of Plasma Kallikrein in Diabetes and Metabolism. *Thromb. Haemost.* 2013, 110 (3), 434–441.

1.2.1 Functions of FXII: Inflammation

BK is an essential mediator for inflammation. The main producer of BK is the plasma contact system in which FXII is the activator. Within the KKS, the binding of BK to the B₂ receptor (Diagram 1) leads to inflammation. This results in dilated vessels, increased vascular permeability, and the induction of chemotaxis in neutrophils¹. When tissue becomes injured or infected, local increases in vascular permeability and controlled inflammation are critical for repair and protection¹⁰.

Closely related to FXII and its inflammatory properties is Hereditary angioedema (HAE). HAE consists of reoccurring severe swelling specifically in the arms, legs, face, and airway. For patients with Type I and Type II HAE there is a deficiency in C1-esterase inhibitor (C1INH). FXIIa activates the fibrinolytic system through PK urokinase activation¹. The main inhibitor for FXIIa and PK is serpin C1 esterase

inhibitor that functions within the contact activation system. Contrastingly, in Type III HAE C1INH functions properly and yet patients still suffer from angioedema. Type III HAE has been shown to be an autosomal dominant disease stemming from a missense, gain of function, mutation on the FXII gene¹¹. A deficiency of FXII can result in thrombo-protection, but excess FXII activity corresponds with HAE with an abundance in BK formation¹². The Madar lab has carried out previous studies to generate an inhibitor of activated FXIIa in order to reduce overexpression of BK.

1.2.2 Functions of FXII: Thrombosis

Venous thromboembolism (VTE) occurs when a blood clot forms, often in deep veins of the legs (Deep Vein Thrombosis). This condition becomes life threatening when the blood clot breaks free from the venous wall and potentially travels to the lungs, resulting in a Pulmonary Embolism (PE). FXIIa induces fibrin formation through FXI in the intrinsic coagulation pathway¹². This contributes to VTE, deep vein thrombosis, PE, ischemic stroke, and atherothrombosis¹². A major cause of death in the Western world, atherothrombosis, produces thrombus formation within blood vessels. The buildup of lipid material within vessel walls can ultimately manifest into coronary artery disease and stroke. Impairing FXII- dependent fibrin formation in ischemic tissue produces thrombo-protective effects¹. This makes drug targets for FXII an attractive mechanism to effectively protect against pathogenic thrombosis without an increased risk of hemorrhage that is a side effect for current anticoagulant drugs¹³.

A prolonged activated partial thromboplastin time (aPTT) tests the speed of clotting. If the clotting takes more than 70 seconds, the aPTT test results show spontaneous bleeding. Interestingly, patients with a FXII deficiency show a prolonged aPTT test but are asymptomatic for bleeding.

The role of FXII in thrombotic disease remains controversial. Some anecdotal evidence, including patient zero, Mr. Hageman, suggest a FXII deficiency increases thrombotic risk factors. In contrast, clinical studies did not find an association with FXII deficiency and thromboembolic disease¹⁴. While this is true, the overall lack of controlled FXII data cannot confirm the presence of thromboprotection in the presence of a FXII deficiency.

When a blood vessel is injured, blood platelets are activated as well as the plasma coagulation system. This induces fibrin formation which produce a blood clot together with platelets. Experiments with the Ross Bengal/laser of vascular injury models and FeCl₃ showed an acute deficiency for thrombus formation in FXII-deficient mice⁸. This challenged the previously accepted coagulation balance¹. FXII is critical for “pathogenic” thrombosis, but it does not play a role in the “physiologic” hemostatic fibrin formation at the site of injury¹. Also controversial is FXII’s role in thrombotic disease. Isolated human trials did not find an association between FXII deficiency and thromboembolic disease¹⁵, even though data supports FXII’s role in thrombosis in mice models¹⁶. Alternatively, epidemiology studies show support for FXII and FXIIa in relation to arterial thrombosis¹⁶. The intrinsic coagulation system shows activation with increased risk for coronary heart disease¹⁶. In patients with ischemic heart disease, studies

show elevated levels of FXII in systemic circulation¹⁷. Experimental stroke models using FXII deficient mice exhibit protection from cerebral ischemia¹⁸. This makes drug targets for FXII an attractive mechanism to effectively protect against pathogenic thrombosis without an increased risk of hemorrhage that is a side effect for current anticoagulant drugs⁸.

1.2.3 Functions of FXII: No Role in Hemostasis

The intrinsic pathway of FXII driven plasma contact system offers promising insight for new research³. FXII promotes the possibility for an anticoagulant target of thrombosis without impacting hemostasis¹. FXII's in vitro role in the plasma contact system is well known, but this enzyme's role in an in vivo setting is still largely unclear. Despite its contribution to fibrin formation in vitro, FXII-initiated coagulation in vivo was not considered to be significant¹. In animal and human models a FXII deficiency does not result in a clinically relevant bleeding phenotype, thus people with FXII deficiency, severe or partial, do not suffer excessive bleeding from the site of injury^{19,20}. The inconsistency with FXII's in vitro and in vivo activity is currently unknown and requires further investigation.

Like FXII deficiency, people with inadequate PK or HK do not suffer hemostasis. Both of these deficiencies are commonly diagnosed during a screening of the activated partial thromboplastin time (aPTT)¹. In contrast, patients who are deficient in FXI suffer from hemophilia C which is a mild bleeding disorder initiated by trauma. Additionally, hemophilia A is caused by a

lack of FVIII, and hemophilia B's source is a tissue factor (TF) deficiency. These observed tendencies suggest that fibrin formation takes place through the extrinsic coagulation pathway²¹. This is confirmed by FVIIa/TF- driven coagulation by the “revised model of coagulation”. This model demonstrates that FXI can activate FXIIa during clotting, independent of FXII²¹. While FXII is dispensable for fibrin formation in hemostasis, future studies are recommended due to the rarity of severe deficiencies²².

1.3.1 Kallikrein: Significance of a Physiological Inhibitor

Plasma kallikrein is a serine protease that cleave peptide bonds and is a key enzyme in the KKS. It functions within this pathway as a combined unit of PK and HK. HK has a molecular weight of 115,000 and combined with PK weighs 285,000²³. PK and HK are associated with a noncovalent bond and act jointly upon FXIIa activation²³. Plasma Kallikrein liberates BK which participates in blood pressure regulation and inflammation activation. Clinically, kallikrein is a drug target for HAE and is a biomarker for multiple types of cancer²⁴. Additionally, plasma kallikrein has been found to be an activator for pro-urokinase which then transforms into its active form, urokinase. This is important in fibrinolysis within the FXII dependent intrinsic pathway¹.

1.3.2 FXII: Significance of a Physiological Inhibitor

FXII is a plasma protein within the intrinsic coagulation pathway. It is also known as the Hageman factor due to its discovery in the first case study. FXII has a

molecular weight of 76,000 daltons and consists of a 596 amino acid glycoprotein¹. FXII is the zymogen form of the protease. Once activated by a negatively charged surface, it becomes FXIIa, a serine protease enzyme. FXIIa participates in the initiation of coagulation, BK production, and fibrinolysis²⁵. Within the KKS, FXIIa activates PK to kallikrein. Then alpha FXII, a form of FXII, causes the activation of FXI in conjunction with HK which participates in thrombosis independent of FXII³. Mice deficient in the B₂ receptor and FXII show a reduced risk for arterial thrombosis²⁶. This suggests a proteolytic system that can reduce arterial thrombosis without affecting hemostasis. Moreover, the KKS can be activated dependent and independent of FXII²⁶.

Currently, drugs targeting blood clotting increase the risk for bleeding. Heparin is a known anticoagulant administered intravenously for venous thrombosis and arterial coagulation. Additionally, Warfarin is an oral anticoagulant with a high affinity for binding plasma proteins. Drawbacks for known inhibitors of the intrinsic coagulation system are slow onset and lack of FXII specificity²⁷. The risks associated with these drugs provides a basis for an improved anticoagulation target that does not increase bleeding.

1.4 History of the Hageman Factor

Fibrin formation functions through two pathways. The extrinsic pathway is initiated by exposure to a damaged blood vessel wall¹. In contrast, the intrinsic pathway responds to blood-borne factors¹. The intrinsic pathway relies on the Hageman factor, FXII, for initiation. The history of this protease dates back to John

Hageman²⁸. He died as a result of pulmonary embolism following a pelvic. After fracturing his left hemipelvis, he was put on bed rest and subsequently allowed to walk. Just days later, Hageman lost a pulse and the ability to breathe which lead to an immediate death. Investigation into his passing revealed a large blood clot impeding flow through both left and right pulmonary arteries as well as multiple thrombi from veins in his lower extremities²⁸. Hageman serves as the index patient for FXII deficiency.

1.5 Summary

A FXII inhibitor has the potential to be an effective anticoagulant drug without affecting hemostasis. The plasma KKS offers a promising research opportunity for therapeutic applications relating to inflammatory thrombolytic diseases³. For this study, we hypothesized that the identification of a specific inhibitor for activated FXII could lead to the reduction of HAE III symptoms and hinder thrombosis formation without an increased risk for bleeding.

RESULTS AND DISCUSSION

2.1 Enzyme Kinetic Determination

Figures 1, 2 and 3 display the optimal physiological concentrations of FXIIa, FXIa and CS-31(02), respectively. The resulting concentrations were then used in all experiments quantifying the inhibition of FXIIa, FXIa and Kallikrein by natural product compounds. The outcome from the chromogenic assays performed indicated that a final concentration of 9 nM FXIIa (Figure 1) successfully hydrolyzed CS-32(02) without oversaturating the absorbance detectors. These experiments were repeated three times and supported by data from other publications. The data from Figure 2 demonstrated that an 18 nM concentration of FXIa should be used in the presence of S2366 for all further experiments. The K_m measures a substrate's ability to interact with an enzyme. Thus, the K_m range for CS-31(02) was determined to be 0.44 to 0.59 mM in the presence of FXIIa (Figure 3). The data also confirmed a 0.43 mM final concentration for CS-31(02) to be used in measurements for FXIIa activity.

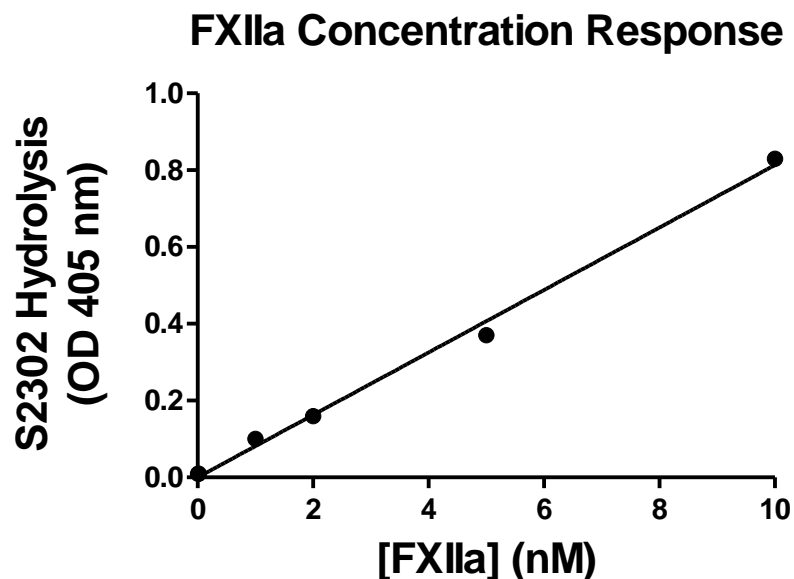


Figure 1: Enzyme Kinetic Determination of FXIIa. FXIIa activity was assayed to determine a consistent concentration of FXIIa to use in the presence of CS-31(02). Due to this experiment, 9 nM FXIIa was used in subsequent experiments to measure changes in FXIIa activity. Two independent experiments were conducted to generate this data. Each experiment consisted of three independent replicates. Mean \pm SEM.

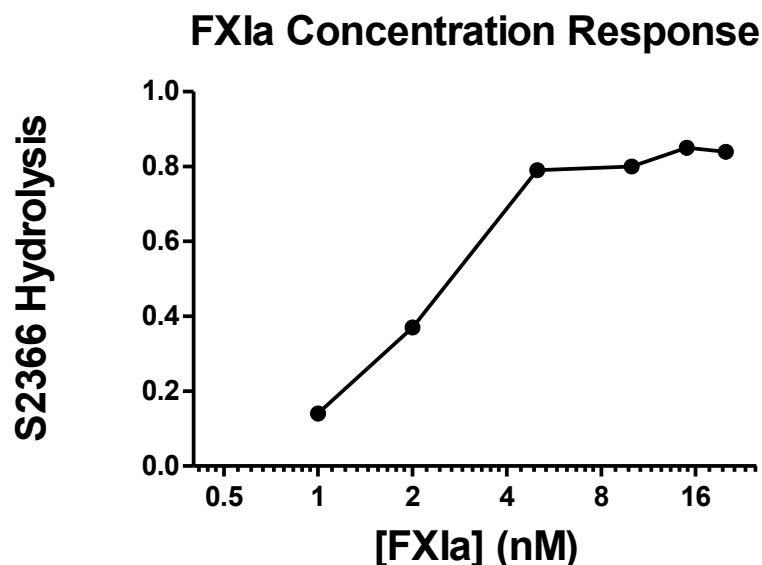


Figure 2: Enzyme Kinetic Determination of FXIa. FXIa activity was assayed to determine a consistent concentration of FXIa to use in the presence of S2366. Due to this experiment, 18 nM FXIa was used in subsequent experiments to measure changes in FXIa activity. One independent study was used for this data. Each experiment consisted of three independent replicates. Mean \pm SEM.

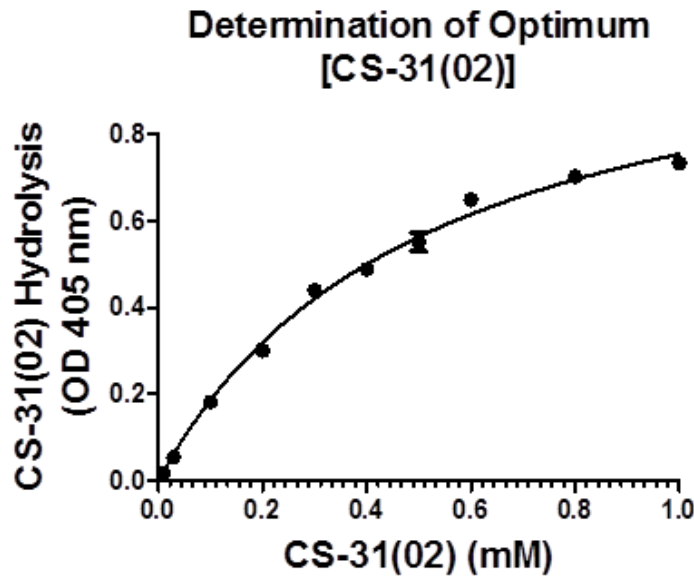


Figure 3: Substrate Kinetic Determination. The K_m range obtained for CS-31(02) in the presence of FXIIa was 0.4375 to 0.5899 mM. For subsequent measurements of FXIIa activity, the final concentration of CS-31(02) equaled 0.43 mM. Three independent experiments were performed. Each experiment consisted of three independent replicates. Mean \pm SEM.

After establishing concentration constants for FXIIa, FXIa and CS-31(02), a substrate time course specification (Figure 4) was completed to quantify the ideal incubation period for chromogenic assays. The time course utilized the previously determined 9 nM FXIIa and 0.43mM CS-31(02). The assay was analyzed at increments of 1, 2, 4, 8, 10, 20, 30, 45, 60, 90 and 120 minuets at OD 405 nm using a BioTek ELx800 Absorbance Microplate Reader. The time course showed an ideal incubation period of 1 hour for all experiments utilizing FXIIa, FXIa, and Kallikrein.

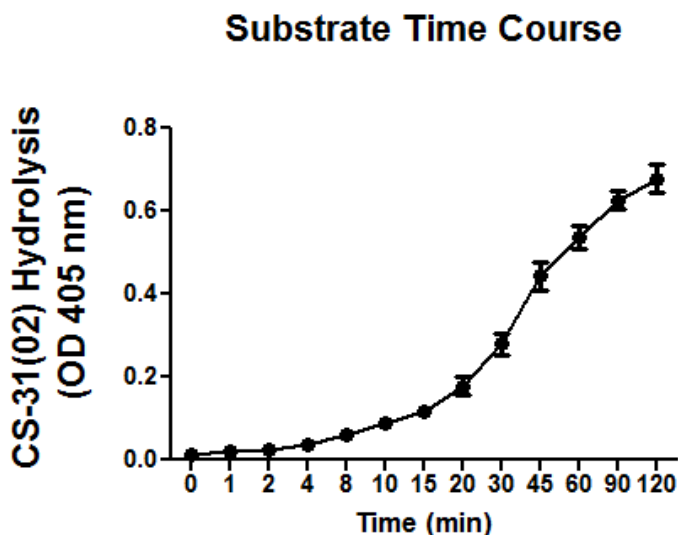


Figure 4: Substrate time course. A time course using 9 nM FXIIa and 0.43 mM CS-31(02) was used to determine the ideal incubation time for the following experiments. Based on this result, an incubation time of 1 hour was used for all experiments utilizing FXIIa, FXIa, and Kallikrein. Three independent experiments in triplicate form generated this data. Mean \pm SEM.

2.2 Determination of Enzyme Inhibition: Myricitrin and Analogues

Myricitrin was one of the original compounds received from the University of Mississippi National Center for Natural Products Research. It was a part of a double-blind study carried out previously by the Madar's laboratory. Once the compound was shown to have inhibitory effects on activated FXII, its identity was disclosed. For the current study, myricitrin and its various analogues were purchased commercially in order to perform further experimentation.

Myricitrin's IUPAC name is 5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-2-(3,4,5 trihydroxyphenyl)chromen-4-one and has a molar mass of 464.37 g/mol. Three independent chromogenic assays were completed in triplicate form to evaluate myricitrin's effect on FXIIa. Findings showed that FXIIa's ability to hydrolyze S2302 was effected by myricitrin. At low concentrations of the

compound, FXIIa showed increased activity in comparison to the control value.

Alternatively, at a concentration of 100 μ M, Myricitrin showed 20-25% inhibition of FXIIa according to mean \pm SEM calculations (Figure 5). This confirmed the results of previous studies, but was not the most successful and specific inhibitor of the desired protease.

For all of the following data representations of concentration dependent experiments on KKS proteases, there was a control used for each protease (FXIIa, FXIa, and kallikrein). Its value was used as a baseline comparison at 100% activity for the effectiveness of inhibitory molecules.

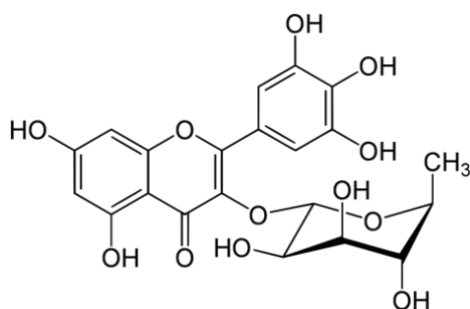
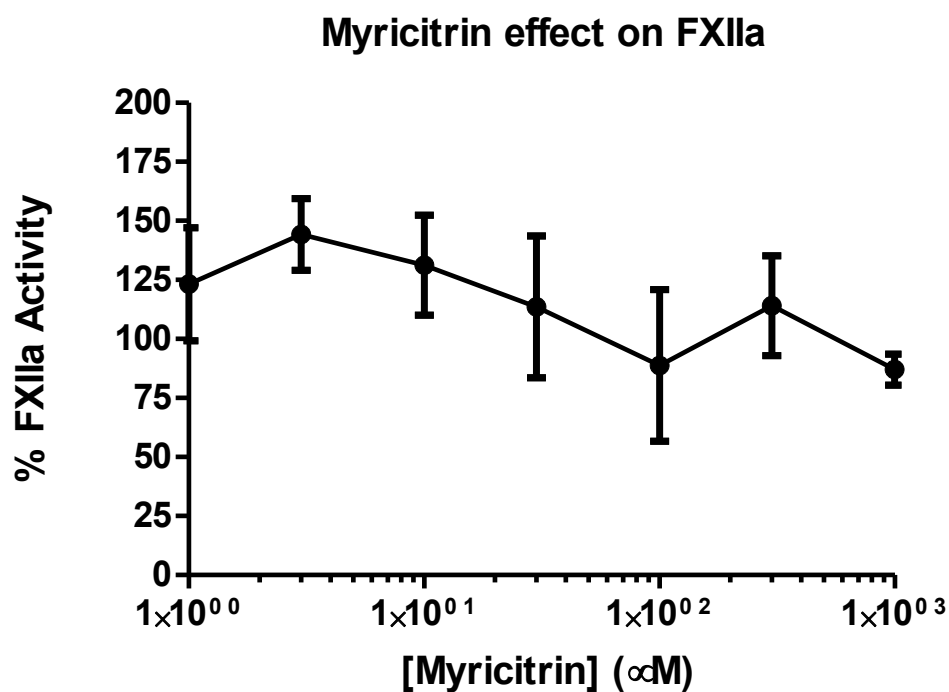


Figure 5: Determination of enzyme inhibition by Myricitrin. This compound was previously tested to show FXIIa inhibition. Three independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

The most promising analogue of myricitrin was myricetin. It is a derivative of myricitrin but lacks an attached saccharide. Myricetin's IUPAC name is 3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone and has a molar mass of 318.23 g/mol. Four independent chromogenic assays were used to test the compounds effect on FXIIa. Results indicated that as the concentration of myricetin increased (1 μ M –

1000 μ M), the inhibitory effects of the compound increased. At a concentration of 1000 μ M, FXIIa was only able to hydrolyze 30% of the substrate in comparison to the controlled S2302 condition, absent of the protease. In conclusion, this data offered favorable inhibitory effects on FXIIa as seen in Figure 6.

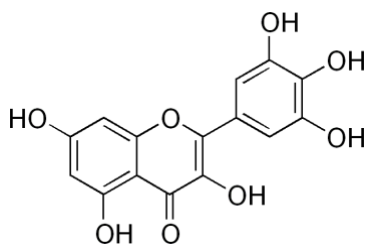
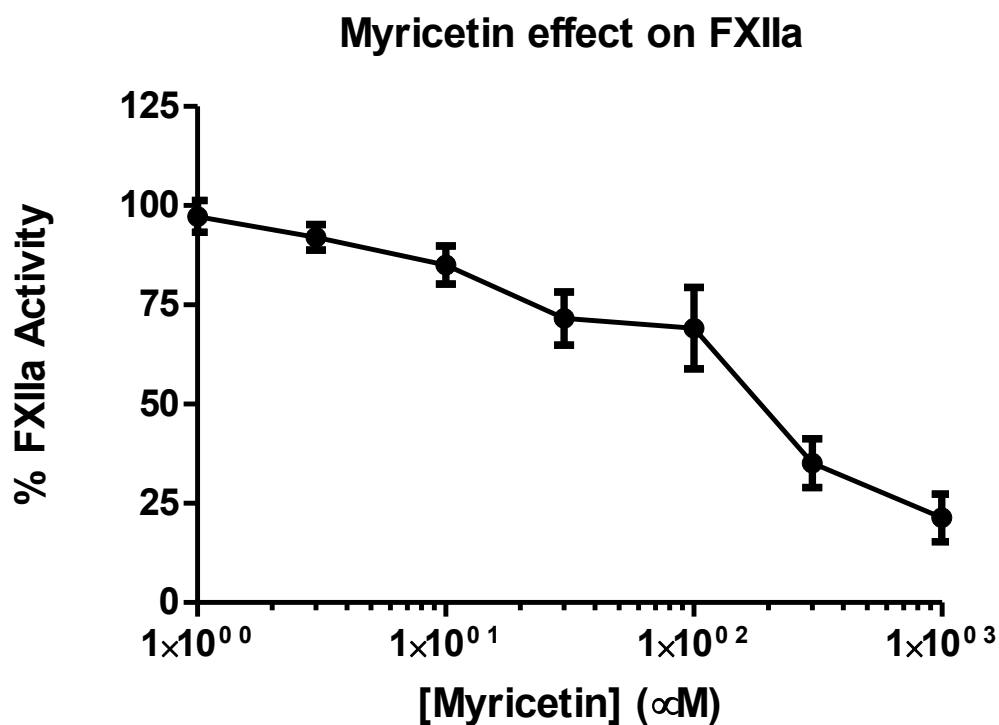


Figure 6: Determination of enzyme inhibition by Myricetin. FXIIa activity was reduced significantly by myricetin. Four independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

Next, myricetin was tested for FXIIa specificity using other proteases from the KKS: FXIa and Kallikrein. Three independent assays showed that myricetin has inhibitory effects on FXIa. A 300 μ M concentration of myricetin blocked 9.56 μ M FXIa at a rate of 70-75% (Figure 7), surpassing its rate of inhibition for FXIIa. Additionally, myricetin blocked Kallikrein activity by 70-80% at a concentration of 30 μ M (Figure 8). Overall, myricetin showed favorable inhibition of multiple proteases within the KKS.

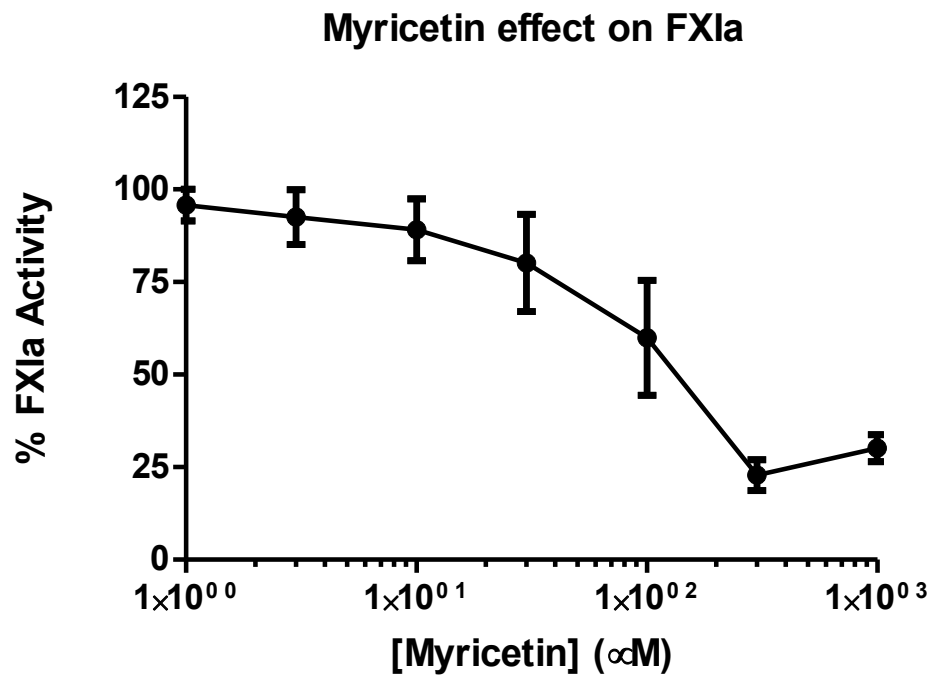


Figure 7: Determination of FXIa inhibition by Myricetin. Three independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

In this study, there was a rise in FXIa activity at a myricetin concentration of 1mM. This does not follow the pattern of the rest of the study. This difference was likely

due to an inconsistency during the dilution phase of the protocol when approaching a higher concentration of myricetin.

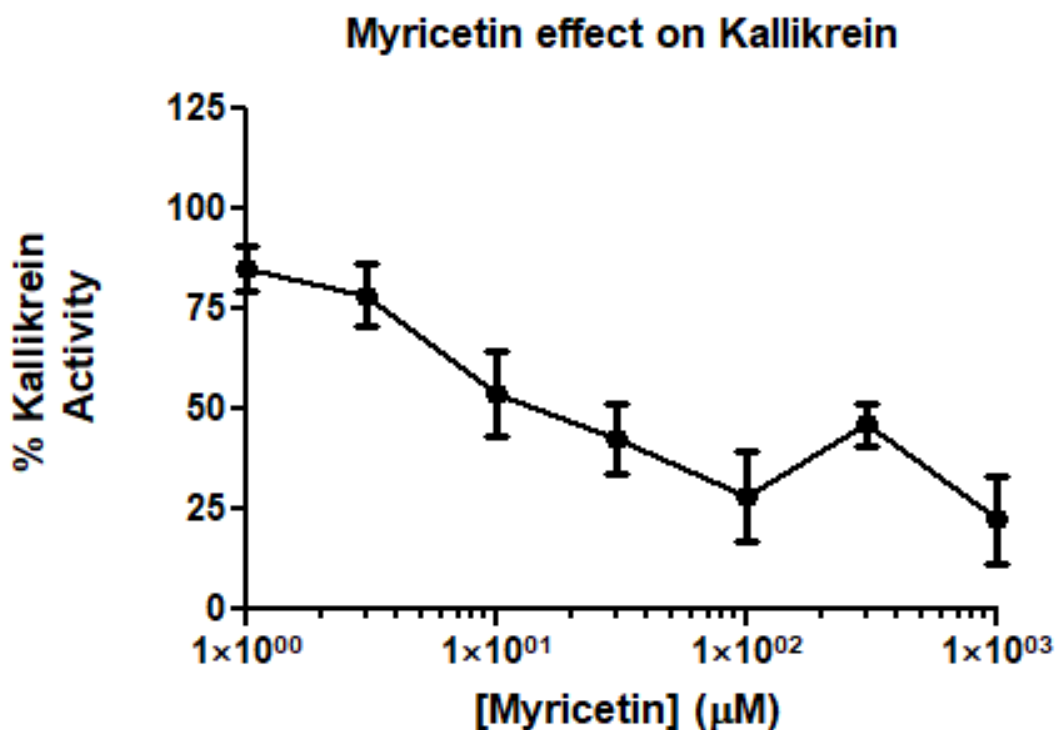


Figure 8: Determination of Kallikrein inhibition by Myricetin. Four independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

In this study, myricetin concentrations between 100 μ M and 1000 μ M showed an inconsistency from the decreasing trend. Because the error bars show overlap with nearby values, this increased value was determined to be not significant in comparison to the overall study.

Another myricitrin analogue tested was quercetin. It differs from myricetin by lacking a hydroxyl group on the 5 position of the phenyl group. Quercetin's IUPAC name

is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one and has a molar mass of 302.23 g/mol. When analyzed in a triplicate chromogenic assay with FXIIa, quercetin reduced FXIIa's ability to hydrolyze S2302 down 30-40% in comparison to the S2302 control group, according to mean \pm SEM data calculations using Graph Pad Software. Because the compound's inhibitory rate was relatively insignificant on FXIIa, further experiments to analyze its specificity to other proteases in the KKS were not preformed.

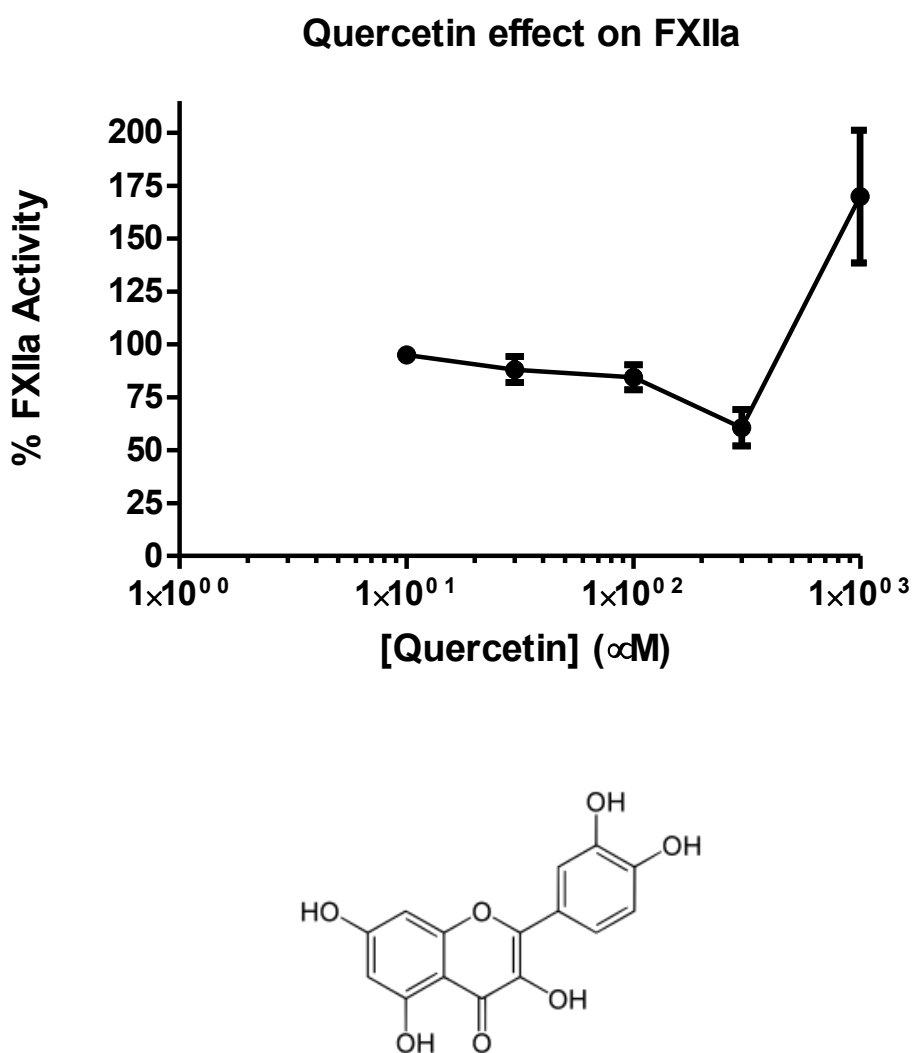


Figure 9: Determination of enzyme inhibition by Quercetin. Quercetin showed minor inhibition of FXII. Three independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

In this set of experiments, there was an inconsistency in the pattern of inhibition as the quercetin concentration reached 1 mM. This was considered to be a molar anomaly. Anomalous behavior has been seen in many drug-protein binding experiments due to complexities with bio-macromolecules and data interpretation²⁹. Further experiments should be done to determine if quercetin is truly an enhancing molecule for FXIIa.

Kaempferol, another analogue of myricitrin, was tested for its effect on active FXII. It differs from myricetin by lacking a hydroxyl on the 3 and 5 position of the phenyl group. Kaempferol's IUPAC name is 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one and has a molar mass of 236.23 g/mol. Three independent assays each in triplicate form were performed to determine kaempferol's effect on FXIIa. A concentration of 1 mM showed the most inhibitory effects on FXIIa, but it was still marginal (20% inhibition) in comparison to other tested compounds that were in lower concentrations. Because the compound did not significantly inhibit FXIIa, it was not analyzed with other proteases within the KKS.

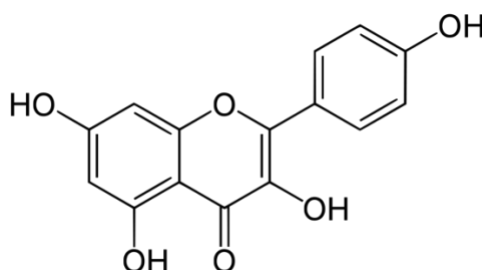
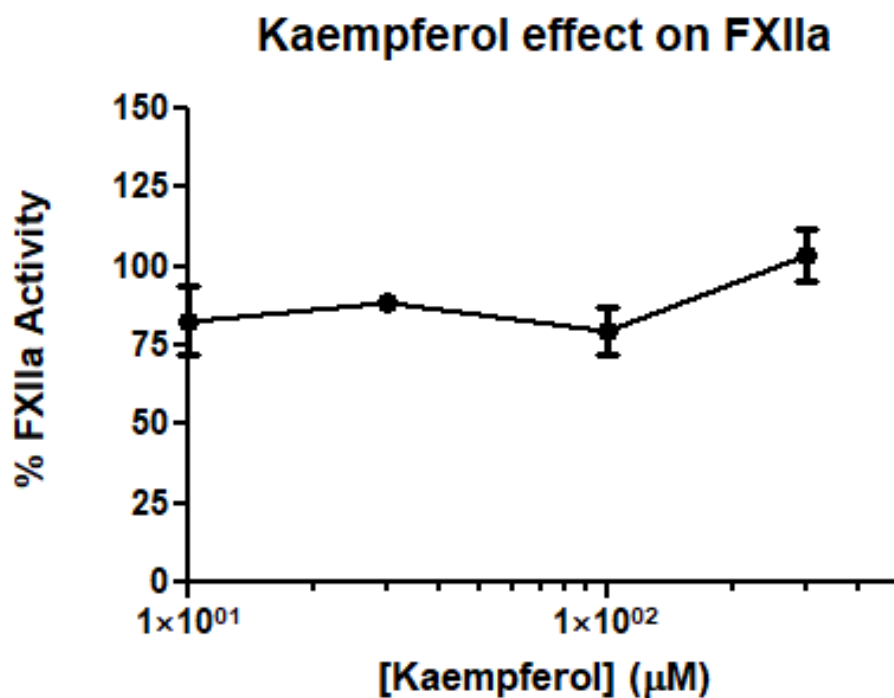


Figure 10: Determination of enzyme inhibition by Kaempferol. Kaempferol showed minor inhibition of FXIIa. Kaempferol is a derivative of myricitrin. Three independent experiments were performed on different days. Each experiment consisted of three independent replicates. Mean \pm SEM.

2.3 Determination of Enzyme Inhibition: Griseofulvin

Like myricitrin, griseofulvin was one of the 196 double-blinded natural product compounds acquired from the University of Mississippi National Center for Natural Products Research. Following Ms. Bensel's identification of griseofulvin as a FXIIa

inhibitor, I worked in conjunction with Chase Suiter to develop and execute further experiments with this compound. This data has been presented by Mr. Suiter at the Ronald E. McNair Post Baccalaureate Program in Baltimore, MD, USA in 2016. Additionally, I displayed our findings at the University of Mississippi Pharmacy School Poster Presentation in August 2016.

Griseofulvin's IUPAC name is (2*S*,6'*R*)- 7-chloro- 2',4,6-trimethoxy- 6'-methyl- 3*H*,4'*H*-spiro [1-benzofuran- 2,1'-cyclohex[2]ene]- 3,4'-dione and has a molar mass of 352.76 g/mol. The compound's structure differs significantly from the myricitrin analogues. Figure 11 demonstrates griseofulvin's effect on 9 nM FXIIa's ability to hydrolyze S2302. Concentrations of griseofulvin ranging from 1 μ M to 3 mM were tested on activated FXII using three independent chromogenic assays in triplicate form. Analysis showed that the IC₅₀ for the compound is 607.0-1192 μ M. This data was based on mean \pm SEM calculations preformed using GraphPad Prism software. At a concentration of 1000 μ M, griseofulvin inhibited FXIIa by 60% in comparison to the control group.

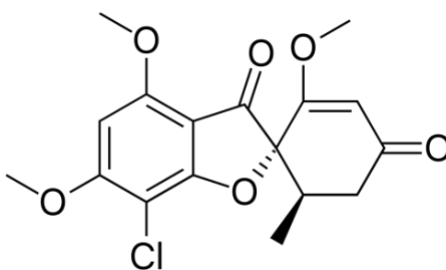
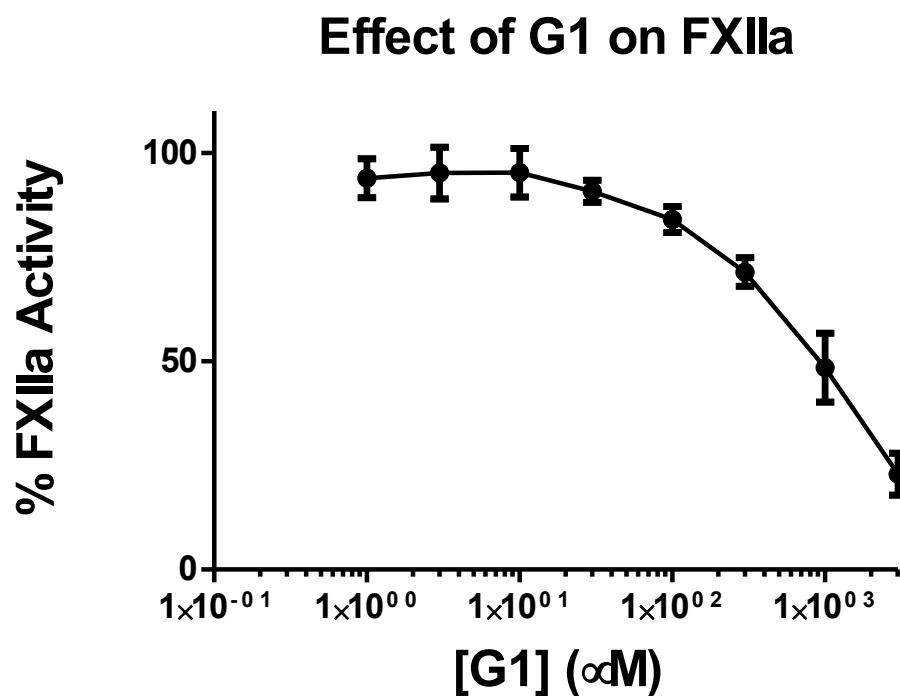


Figure 11: Determination of FXIIa inhibition by Griseofulvin. This compound was previously tested to show FXIIa inhibition. Three independent experiments were performed on different days. Each experiment consisted of three independent replicates. Mean \pm SEM.

Because griseofulvin was confirmed to be a promising inhibitor of FXIIa, its specificity was tested on other proteases in the KKS. The IC_{50} for griseofulvin in the presence of FXIa was 2.886 μ M (Figure 12). The compound inhibited FXIa activity at a slightly lower rate than it did FXIIa. Griseofulvin was also tested in the presence of Kallikrein, and it did not show inhibition. To confirm that the Kallikrein used in Figure

12 had not lost its biological activity, Kallikrein was incubated with Kallistop, a well know synthetic inhibitor of Kallikrein. Concentrations of Kallistop ranged from 0.1 μM to 300 μM , with the 300 μM Kallistop showing ~3% Kallikrein activity. This finding confirmed that compound G1 does not inhibit Kallikrein (Figure 13). Overall, griseofulvin inhibited FXIIa and FXIa with little effect on Kallikrein (Figure 14). In comparison to myricetin, griseofulvin was not shown to inhibit FXIIa at a comparable rate.

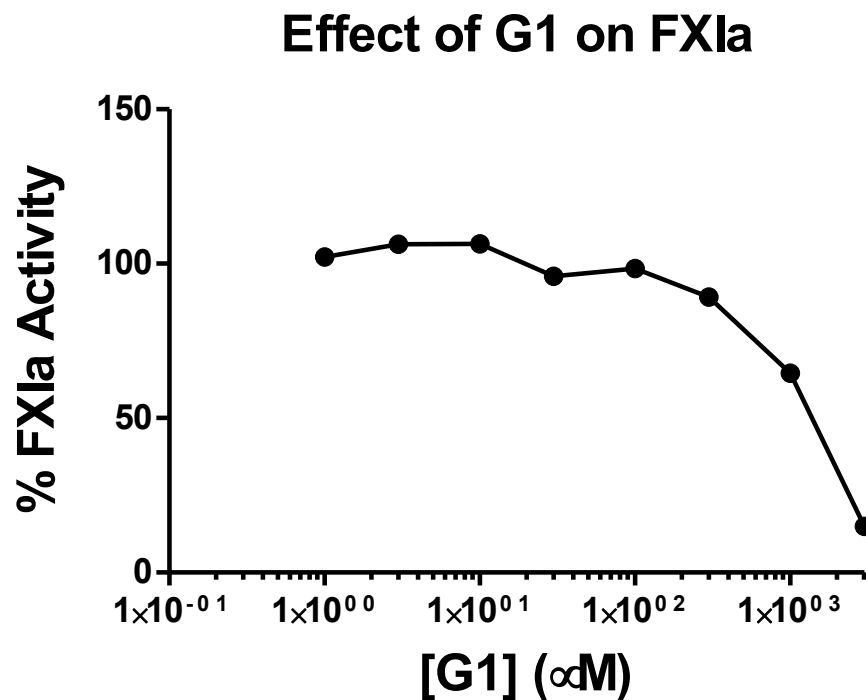


Figure 12: Determination of FXIa inhibition by Griseofulvin. This compound showed inhibition of FXIa. One independent experiment was performed. It consisted of three independent replicates. Mean \pm SEM.

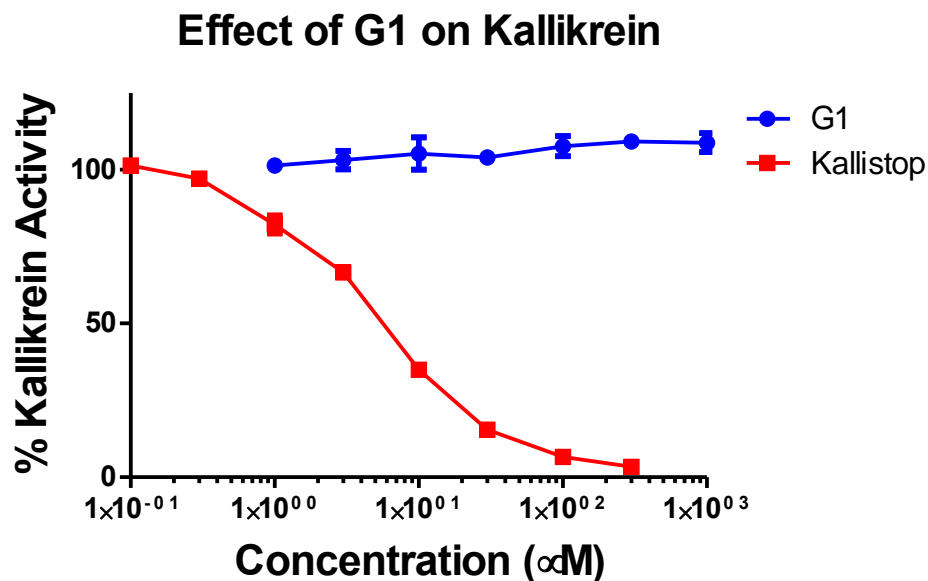


Figure 13: Determination of Kallikrein inhibition by Griseofulvin. This compound did not show inhibition of Kallikrein, especially in comparison to its known inhibitor Kallistop. Three independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

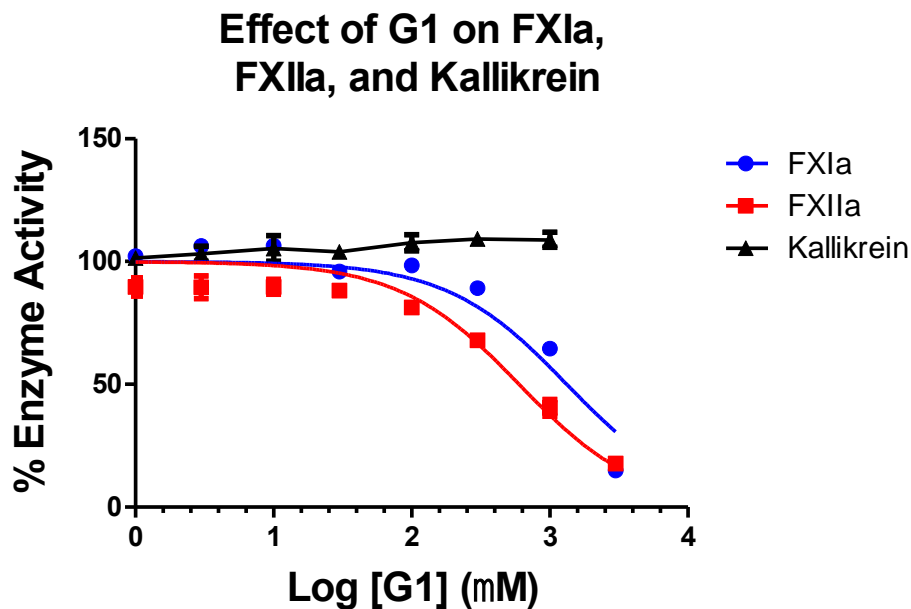


Figure 14: Comparison of Griseofulvin specificity to FXIIa, FXIa and Kallikrein. This figure combines independent experiments with FXIa, FXIIa, and Kallikrein to properly compare their effects. Mean \pm SEM.

2.4 Determination of Enzyme Inhibition in Human Plasma

All previous experiments were performed in a pure system. The objective of subsequent experiments was to determine if the same inhibitory effects could be duplicated in plasma. To mimic human physiological conditions, FXIIa hydrolysis of its specific substrate, CS-31(02), was measured in the presence of human plasma. Human plasma serves as the main medium in which plasma proteins function. Figure 15 shows the results of a chromogenic triplicate assay with FXIIa concentrations ranging from 0.001 nM to 2 nM. The activity of the protease grows proportionality with increasing concentration according to measurements taken at OD 405 nm. Data are presented as mean \pm SEM.

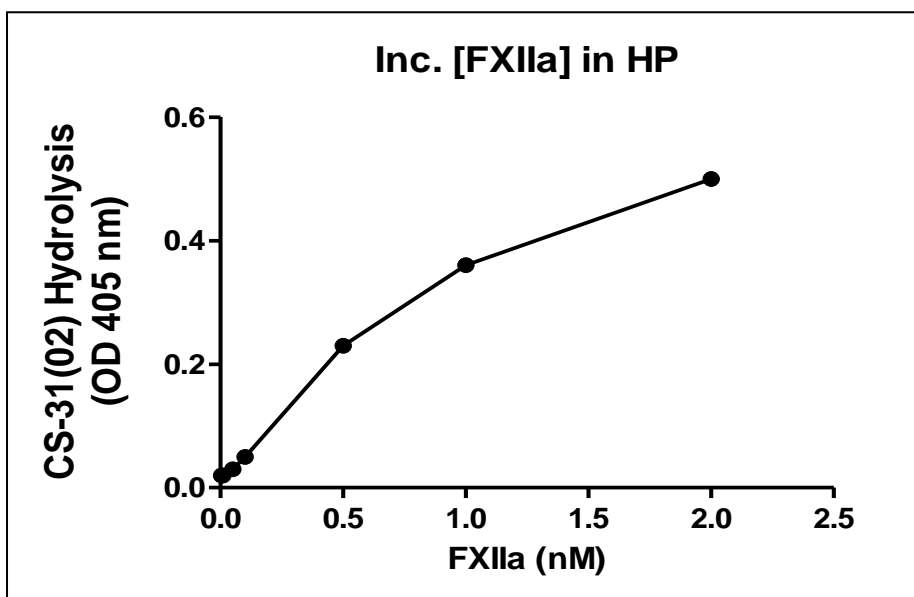


Figure 15: Analysis of increasing concentrations of FXIIa in human plasma. One independent study was used for this data. Each experiment consisted of three independent replicates. Mean \pm SEM.

Next, the inhibitory compounds were evaluated in conjunction with the KKS proteases in the presence of human plasma. Figure 16 depicts the findings for griseofulvin's effect on FXIIa. As a control group, a known inhibitor Kallistop (KS) was used as a comparative control. Kallistop reduced FXIIa's ability to hydrolyze S2302 shown by the second bar. In the presence of normal human plasma (NP), KS was also able to significantly inhibit the activity of FXIIa. Alternatively, the addition of griseofulvin (Gris) to the system caused an increase to active FXII's rate of hydrolysis. In conclusion, this did not mimic griseofulvin's effect on FXIIa in a pure system.

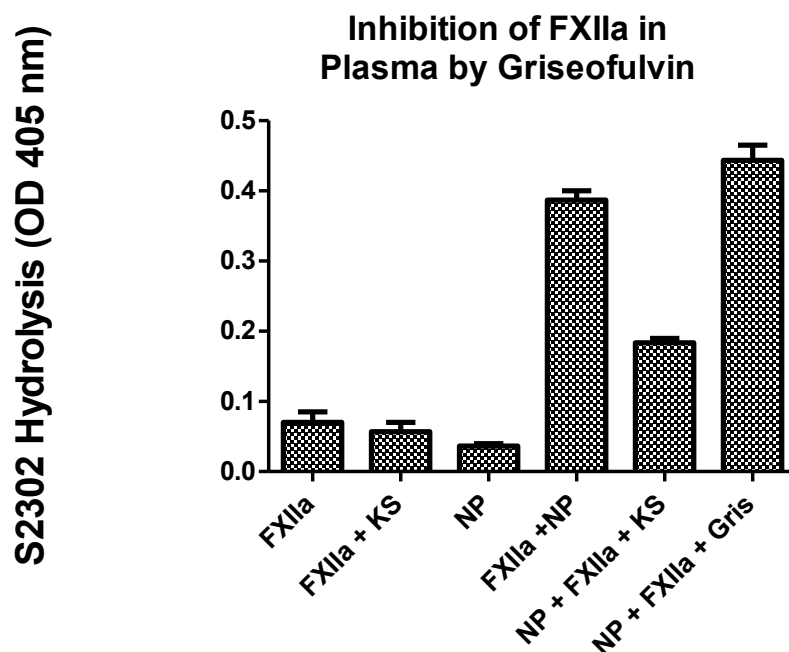


Figure 16: Inhibition of FXIIa in human plasma by Griseofulvin. Three independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

The effect of myricitrin was then tested on KKS proteases in the presence of human plasma. For this characterization in plasma, FXII was intentionally activated in order to properly evaluate the compound in the presence of FXII. Because FXII circulates

in its zymogen form, the addition of FXIIa was essential in order to draw accurate conclusions about FXII's activity. Figure 17 shows the effect of myricitrin (Myr) on kallikrein using the known inhibitor KS as a control. In an isolated system myricitrin reduced the activity of kallikrein. Alternatively, in the presence of NP, myricitrin stimulated the activity of kallikrein. Overall, myricitrin did not show specificity to kallikrein in the presence of human plasma.

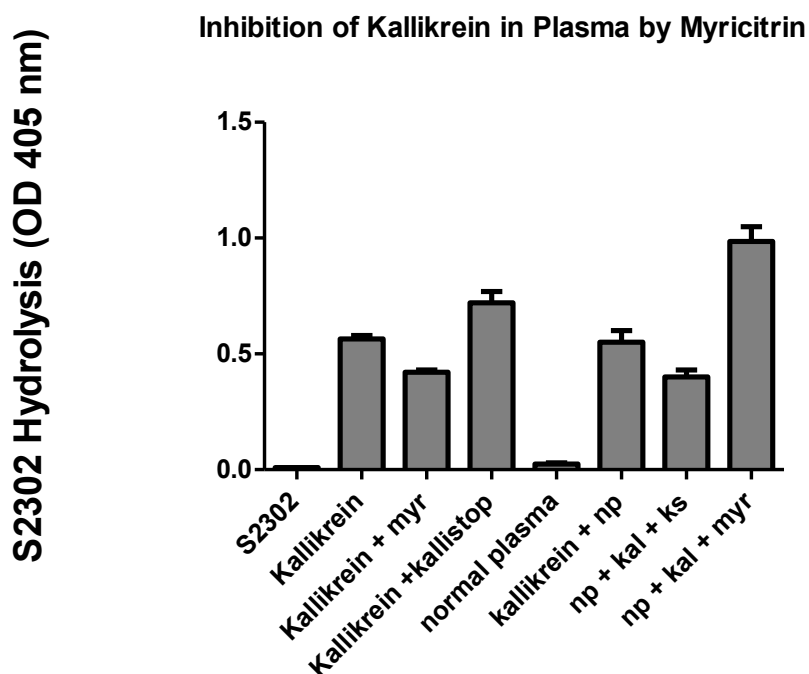


Figure 17: Inhibition of Kallikrein in human plasma by myricitrin. Three independent experiments were performed at different times. Each experiment consisted of three independent replicates. Mean \pm SEM.

Further experimentation should be performed to evaluate myricetin's effect on FXIIa, FXIa, and Kallikrein in a system with human plasma. The results of the compound's effect on the KKS proteases are promising in a pure system. Ideally,

myricetin would perform the same inhibitory actions in an environment mimicking human physiological conditions.

CONCLUSION

This study evaluated the effects of natural product compounds on FXIIa and other Kallikrein Kinin-System proteases. The findings indicated promising drug development opportunities. Myricetin showed the strongest inhibitory effects and specificity towards FXIIa. Griseofulvin and myricitrin also prevented FXIIa's ability to hydrolyze S2303 but at a lesser rate. Finally, myricitrin's analogues quercetin and kaempferol had little to no effect on FXIIa activity. These findings in both a pure system and in plasma offer an attractive target specifically blocking FXIIa. Myricetin has the potential to be a useful multifunctional therapeutic drug in patients with Hereditary Angioedema and thrombolytic diseases.

Finding a specific activated FXII inhibitor presents the possibility to combat a variety of severe health conditions including Hereditary Angioedema and thrombolytic diseases. Pathogenic thrombosis stemming from the plasma coagulation system has the capability to be effectively combated through drug targets for FXII dependent mechanisms. Potentially, this could impair FXII dependent fibrin formation without the associated risk of hemorrhage. FXII is also a target for diseases associated with inflammation that interact with the Kallikrein Kinin-System. Hereditary Angioedema Type III generates excess bradykinin that produces life threatening swelling effects. Inhibiting activated FXII could prematurely block the binding of bradykinin to B2 receptors and successfully reduce proinflammatory signaling. These health conditions

currently lack an effective inhibitory drug served as the basis for this study. Myricetin offers a comprehensive range of positive effects due to its ability to inhibit FXII.

Further experiments are required in the field of computational biology in order to properly assess and explore the interaction of myricetin with crystals of FXIIa. Compound selectivity is the foundation of a true protease inhibitor. The chemical structure of myricetin offers insight into what specifically inhibits the binding region of activated FXII. An evaluation of this crystal structure would permit an in-depth study into the relationship of FXIIa and the inhibitor.

Current conditions affected by FXII lack specific and potent drug targets. There is weakness in the field of known anticoagulants due to their association with increased bleeding risks. A FXII inhibitor could be an effective anticoagulation drug without affecting hemostasis. Additionally, Hereditary Angioedema Type III lacks a specific inhibitor. The results of this study offer a hopeful method to combat HAE Type III, which currently has no established drug market. This study shows that myricetin offers promising opportunities for therapeutic significance in health conditions associated with over-activation of the Kallikrein Kinin-System.

EXPERIMENTAL DESIGN AND METHODOLOGY

Background

This study is an expansion of experiments designed and executed by Alexandra Bense (*Identification of Inhibitors of Factor XII*, May 2016) under the direction of Dr. Ziaeddin Shariat-Madar. Her focus was on Bradykinin's role in Type III Hereditary Angioedema. The goal of the study was to identify potential inhibitors of FXII in order to reduce FXII expression and bradykinin production. Ideally, this would alleviate severe symptoms of Type III HAE. She tested 176 double blinded natural product molecules in attempt to identify inhibitors. My studies focus on two of the natural products later identified that showed significant inhibition of FXII *in vitro*.

Materials

The Madar lab received two 96 well plates from The University of Mississippi National Center for Natural Products Research. The plate identifications were NCNPR-XTL-036 and NCNPR-XTL-O47. Each plate contained samples from 88 molecules and all were tested in conjunction with FXII. Human Factor alpha-XIIa, Human Factor alpha-Xia, and Plasma Kallikrein were purchased from Enzyme Research Laboratories, South Bend, IN, USA. The Human Factor alpha-XIIa stock concentration was 1.29 mg/ml with activity of 73.57 PEU/mg. To activate the Human Factor alpha-XIIa from its homogenous form, Dextran Sulfate was used and then taken out following activation. The

effect of the inhibitory compounds on FXIIa and Kallikrein were determined using the chromogenic substrate S-2303, also referred to as CS-31(02). It was purchased in a 4 mM concentration from Aniaara Diagnostica, West Chester, OH, USA. For experiments dealing with compound specificity to FXIa, S-2366 (Glu-Pro-Arg-p-nitroanilide) was used as the substrate. It was purchased in a 4 mM concentration from DiaPharma Group, Inc. Inhibitory compounds griseofulvin and myricitrin analogues were purchased from Sigma-Aldrich (St. Louis, MO, USA). The plasma proteins were incubated with their respective enzymes in the presence of inhibitory compounds and HEPES-NaHCO₃ buffer. The buffer solution was made in the lab using 137 mM NaCl, 3 mM KCl, 12 mM NaHCO₃, 14.7 mM HEPES, 5.5 mM glucose, 0.1% gelatin, 2 mM CaCl₂, and 1 mM MgCl₂. The pH was then adjusted to 7.1 using HCl and NaOH. Following incubation, the chromogenic FXIIa activity assay was measured as change in absorbance at OD 405 nm using BioTek ELx800 Absorbance Microplate Reader (Winooski, VT, USA). Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Methods Overview

The effect of griseofulvin and myricetin, new inhibitors of plasma enzymes, were characterized by chromogenic assay in the presence of FXIIa, FXIa, and kallikrein. Enzymes and peptides were always put on ice. Leaving them at room temperature could result in premature activation. Additionally, all pipet tips were autoclaved before use. In these experiments, 9 nM FXIIa was incubated with known chromogenic substrate S-2302 that is selective for FXIIa. S-2302 is hydrolyzed by FXIIa. This process cleaves a peptide bond between arginine and para nitroaniline (pNA), with pNA resulting in a yellow color

change. A darker solution indicates that more of the substrate (S2302) is being hydrolyzed by FXIIa. FXIa and kallikrein were measured similarly, using 0.3 mM S2366 and 0.4 mM S-2302, respectively. Plasma proteins were incubated with their respective enzyme in the absence or presence of increasing concentrations of potentially inhibitory natural product compounds with a final volume of 330 μ L. The incubation parameters remained constant throughout all experiments at 37°C and 1 hour. The change in absorbance was measured based on the production of free para-nitroaniline at OD 405 nm.

Concentration Response: FXIa and FXIIa

The objective for this experiment was to determine the optimum FXIa concentration to use for inhibition analogues. A FXIa and S2366 control was used as a baseline comparison for the dependent variable. The FXIa control contained only FXIa, HEPES buffer (pH: 7.1), and S2366. The S2366 negative control only contained HEPES buffer and the respective substrate. The conditions included a varying concentration of the FXIa (1,2,5,10,10,20 nM). HEPES buffer (pH: 7.1) was used to obtain a final volume of 330 μ L. Lastly, a constant 0.3 mM of S2366 was added to each well quickly but without compromising the integrity of the experiment. Following the addition of the substrate, the tubes were gently pipetted in and out to mix the sample. The sample was then transferred in 100 μ L increments to triplicate wells of an assay plate. The plate was incubated at a temperature of 37°C for 1 hour. The change in absorbance was measured at OD 405 nm to determine optimum FXIa activity. Triplicate data was taken from four independent experiments using mean \pm SEM.

For FXIIa, an identical methodology was used with minor changes. The substrate specific for FXIIa is S2302, and it was used in a 0.37 mM concentration. Additionally, the concentrations for FXIIa were expanded to 0.01, 0.02, 1, 2, 5, and 10 nM.

Dose Response: S2302 (CS-13(02))

The objective of the CS-13(02) dose response was to determine the K_m of the substrate in the presence FXIIa. A constant 9 nM FXIIa was used in the experiment. The concentration of S2302 varied (10,30,100,200,300,400,500,600,800,1000 μ M).

Additionally, HEPES bicarbonate buffer was added to obtain a final volume of 330 μ L in each sample tube. Following the addition of the substrate, the tubes were gently pipetted in and out six times to mix the sample. The sample was then transferred in 100 μ L measurements to triplicate wells of an assay plate. The plate was incubated at a temperature of 37°C for 1 hour. The change in absorbance was measured at OD 405 nm to determine optimum FXIa concentration. Triplicate data was taken from four independent experiments using mean \pm SEM.

CS-13(02) Time Course

The objective of the CS-13(02) time course was to determine the ideal incubation time for the following experiments. Activated 9 nM FXII was used along with 0.43 mM CS-13(02) substrate. HEPES buffer (pH: 7.1) was used to obtain a final volume of 330 μ L. Following the addition of the substrate, the tubes were gently pipetted in and out to mix the sample. The sample was then transferred in 100 μ L measurements to triplicate wells of an assay plate. The plate was put in the incubator at 37°C for 1 hour. It was

removed from the incubator and analyzed at 1, 2, 4, 8, 10, 15, 20, 30, 45, 60, 90, and 120 minutes at OD 405 nm.

Inhibition of Plasma Proteases by Myricitrin and its analogues

The objective of this experiment was to determine the effect of myricitrin on FXIIa. The conditions included a varying concentration of the inhibitory compound (1,3,10,30,100,300,1000 μ M). A set of negative controls, lacking FXIIa, of equivalent concentrations were used to account for the color present in myricitrin. This control removed the chance of obtaining false positive results from the BioTek ELx800 Absorbance Microplate Reader. Also, FXIIa and CS-13(02) controls were used as a baseline comparison for the dependent variable. The FXIIa control contained only FXIIa, HEPES buffer (pH: 7.1), and CS-13(02). The CS-13(02) negative control only contained HEPES buffer and the respective substrate. A constant 0.43 mM of S2302 was added to each sample tube after 9 nM activated FXII. HEPES buffer (pH: 7.1) was used to obtain a final volume of 330 μ L. Following the addition of the substrate, the tubes were gently pipetted in and out to mix the sample. The sample was then transferred in 100 μ L measurements to triplicate wells of an assay plate. The plate was incubated at a temperature of 37°C for 1 hour. The change in absorbance was measured at OD 405 nm to determine the rate of FXIIa inhibition by myricitrin. Triplicate data was taken from four independent experiments using mean \pm SEM.

For myricitrin's effect on Kallikrein, an identical protocol was used and simply replaced FXIIa with Kallikrein. For myricitrin's effect on FXIa, S2366 was used as the substrate at a final concentration of 0.3 mM, and the remaining parameters were kept

constant with the other plasma protease experiments. The same protocol was used for experiments using myricitrin and quercetin, both analogues of myricitrin. To see inhibition for kaempferol and quercetin, also analogues of myricitrin, concentrations for the compounds were expanded to 0.01, 0.03, 0.1, 1, and 3 mM. The other variables were kept uniform in order to properly compare the effects of differing analogues.

Inhibition of Plasma Proteases by Griseofulvin

The purpose of this experiment was to determine the effects of griseofulvin on FXIIa. 9 nM activated FXII was used along with 0.4 mM S2302. FXIIa and S2302 controls were used as a baseline comparison for the dependent variable. The FXIIa control contained only FXIIa, HEPES buffer (pH: 7.1), and S2302. The S2302 negative control only contained HEPES buffer and the respective substrate. The concentrations of griseofulvin were varied at 1, 3, 10, 30, 300, 1000, and 3000 μ M. HEPES bicarbonate buffer (pH 7.1) was added to give each mixture a final volume of 330 μ L. The substrate was added last and in a time efficient manner. Then, each tube was pipetted in and out to mix the solution. The sample was transferred in 100 μ L measurements to triplicate wells of an assay plate. The plate was incubated for 1 hour at 37°C. The change in absorbance was measured at OD 405 nm to determine the rate of FXIIa inhibition by griseofulvin. Triplicate data was taken from three to four independent experiments using mean \pm SEM.

Activated FXII in Human Plasma

The objective of this study was to measure FXIIa activity in normal plasma. FXIIa and S2302 controls were used as a baseline comparison for the dependent variable.

The FXIIa control contained only FXIIa, HEPES buffer (pH: 7.1), and S2302. The S2302 negative control only contained HEPES buffer and the respective substrate. HEPES buffer was added to each tube to obtain a final volume of 330 μ L. Lastly, S2302 was added with a final concentration of 0.3 mM. Following the addition of the substrate, the tubes were gently pipetted in and out to mix the sample. The sample was then transferred in 100 μ L measurements to triplicate wells of an assay plate. The plate was incubated at a temperature of 37°C for 1 hour. The change in absorbance was measured at OD 405nm to determine FXIIa activity in normal and deficient human plasma. Triplicate data was taken from three to four independent experiments using mean \pm SEM.

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